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A New Chromatographic Procedure for Isolation of Lipoprotein Fractions

James Quinn Kissane
Loyola University Chicago

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**A NEW CHROMATOGRAPHIC PROCEDURE
FOR ISOLATION OF LIPOPROTEIN FRACTIONS**

by

James Quinn Kissane



**A Thesis Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Master of Science**

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1962

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LIFE

James Quinn Kissane was born in Oak Park, Illinois on October 27, 1932. After graduating from Fenwick High School, Oak Park, Illinois, he attended Loras College, Dubuque, Iowa and received the Bachelor of Science in chemistry in June, 1955.

He was inducted into the U.S. Navy in November, 1955 and served 21 months as a personnelman in the information and education field.

Upon receiving an honorable discharge in August, 1957, he was employed as a chemist by the Glidden Paint Company and assigned to a position in quality control at the Nubian Division, Chicago, Illinois.

In January, 1959, he began his graduate curriculum in biochemistry at Loyola University and held the position of Research Assistant, which was supported by the Chicago and Illinois Heart Association until July, 1960. At this time he was employed by Cook County Hospital as a member of the staff in the Department of Biochemistry.

Mr. Kissane is coauthor of the following publications:
"The Separation of Human Serum Lipoproteins by Conventional Descending and Centrifugally Accelerated Paper Chromatography", Anal. Biochem., 1, 44-59 (1960) and "Sudan Black B in Ethyl Acetate - Propylene Glycol for Prestaining Lipoproteins", Anal. Biochem., 1, 178-179 (1960)

TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF FIGURES	vi
CHAPTER	
I. INTRODUCTION	1
Introduction--Physical and chemical fractionation methods--Chemical composition of serum lipopro- teins--Biochemical aspects of human serum lipo- proteins--Clinical aspects of serum lipoproteins-- Theory of chromatography--Problems of this in- vestigation.	
II. MATERIALS AND METHODS	35
Human Serum--Prestaining technique--Ultracentri- fugal fractionation of serum lipoproteins--Des- cending paper chromatography--Paper ionography of serum lipoproteins--Densitometric determin- ations.	
III. FRACTIONATION OF SERUM LIPOPROTEINS BY CENTRI- FUGALLY ACCELERATED CHROMATOGRAPHY	51
IV. RESULTS AND CONCLUSIONS	56
V. SUMMARY	71
APPENDIX	72
BIBLIOGRAPHY	77

LIST OF TABLES

TABLE	Page
I. PROPERTIES AND COMPOSITION OF THE FIVE PLASMA LIPOPROTEIN FAMILIES	9
II. AMINO ACID COMPOSITION OF THE PLASMA LIPOPROTEINS	11
III. ULTRACENTRIFUGAL FRACTIONS OF SERUM LIPOPROTEINS	42
IV. R_B VALUES OF LIPOPROTEIN FRACTIONS	58
V. STAIN INTENSITY VS. LIPID:DYE CONCENTRATION TEN MICROLITER ALIQUOT	59
VI. STAIN INTENSITY VS. LIPID:DYE CONCENTRATION TWENTY MICROLITER ALIQUOT	60
VII. BEER'S LAW CONSTANTS FOR VARYING LIPID:DYE CONCENTRATIONS	64
VIII. DENSITOMETRIC ANALYSIS OF SERUM LIPOPROTEINS	68

LIST OF FIGURES

FIGURE	Page
1. RELATIONSHIP OF LOG OF STAIN INTENSITY TO PER CENT CONCENTRATION OF PRESTAINED SERUM TEN MICROLITER ALIQUOT	61
2. RELATIONSHIP OF LOG OF STAIN INTENSITY TO PER CENT CONCENTRATION OF PRESTAINED SERUM TWENTY MICROLITER ALIQUOT	62
3. PHOTOGRAPH OF CHROMATOFUGE, EXTERIOR ASPECTS	73
4. PHOTOGRAPH OF CHROMATOFUGE, INTERIOR ASPECTS	74
5. PHOTOGRAPH OF A LIPOPROTEIN CHROMATOGRAM DEVELOPED BY CENTRIFUGALLY ACCELERATED CHROMATOGRAPHY	75
6. PHOTOGRAPH OF A MODIFIED LIPOPROTEIN CHROMATOGRAM DEVELOPED BY CENTRIFUGALLY ACCELERATED CHROMATOGRAPHY .	76

CHAPTER I

INTRODUCTION

Lipoproteins are conjugated proteins composed of peptide chains and a variety of lipid molecules. Such an entity is not only an interesting academic curiosity, but also a biologic necessity. Physical and analytical study have not only proven the existence of these complex moieties, but also they have shed some light on the mechanism of lipid transport, lipid exchange and cellular structure. Lipoproteins have been investigated with respect to their formation and to their possible role in disease states, most notably in atherosclerosis; and although this subject is still highly controversial, it is noteworthy that an alteration of the serum lipoproteins does exist. At this stage of development it is difficult to project the exact significance of the lipoproteins in health and disease states, but it would be presumptuous to discount or to unduly exaggerate their relationship to biologic processes, both normal and pathological. A great deal of study remains in the area of lipid metabolism before any precise evaluation of the lipoproteins can be given.

Isolation of serum lipoproteins has been carried out by employing various physical factors, most notably density, electrophoretic mobilities, fractionation methods involving either cold ethanol-water mixtures or ammonium sulfate, and chemical interaction with sulfated polysaccharides.

PHYSICAL AND CHEMICAL FRACTIONATION METHODS

The first reported separation of a lipoprotein was that by Macheboeuf in 1928 (36), in which one of the soluble protein fractions remaining in a one-half saturated ammonium sulfate solution at neutral pH was precipitated by adjusting the solution pH to 3.8. The acidic precipitation was repeated several times until a purified protein fraction was isolated. The horse serum fraction thus isolated was soluble in water at neutral pH and exhibited a remarkable constancy in composition. The protein contained no free cholesterol and was found to be present in horse serum at a concentration of 2.5 grams/liter. Later Macheboeuf determined that the lipoprotein fraction possessed the mobility of an alpha-1-globulin during electrophoresis (37).

By taking advantage of solubility properties of plasma proteins, Cohn and his coworkers (17) were able to isolate two distinct lipoprotein fractions from human plasma. It had been demonstrated that small amounts of electrolytes or larger amounts of glycine would, in general, increase the solubility of proteins; while the introduction of ethanol would decrease the solubility.

This alcohol fractionation method was conducted at a temperature of -5 degrees centigrade, in order to enhance the precipitation of the more insoluble fractions and also to minimize denaturation of the proteins. By progressively altering the ionic strength, the pH and the ethanol concentration of the solution, plasma proteins could be selectively precipitated. The alpha and the beta fractions of lipoproteins were thus isolated.

A study of the beta lipoproteins revealed that it contained seventy-five per cent of the plasma lipid and represented five per cent of the total plasma protein. This method was later modified and improved in 1950 (18) by reducing the concentration of ethanol necessary for fractionation. This modification was introduced in order to decrease the denaturing effect of the organic solvent.

Several serious limitations are inherent in the foregoing technique; one of which is the extensive amount of lipid free protein which is coprecipitated with the beta lipoprotein fraction, and the other is the possible extraction of appreciable quantities of lipid from the lipoprotein which would alter the analysis of such fractions and thereby reflect a composition entirely different from that which occurs in its native state.

A specific interaction with beta lipoprotein was demonstrated by Bernfeld (5) in 1958, in which the quantity of this protein fraction could be determined nephelometrically. Macro polysaccharide sulfate esters were shown to interact with plasma

proteins and form an insoluble complex at pH 7 and lower; however by controlling the pH at 8.6, the interaction became specific for beta lipoproteins. A modification of the procedure was introduced whereby the initial serum sample was diluted 250 times its original concentration in order to produce a finely dispersed suspension of the lipoprotein-polysaccharide complex which could then be easily measured by the nephelometric method. In 1960 Bernfeld (6) reported the use of amylopectin, derived from Corn starch and sulfated according to the method of Schoch (54), in a broad study of lipoprotein levels in normal and atherosclerotic populations. The utilization of a commercial Nephelos standard was reported. By employing a factor of 12.1 to the nephelos index, Bernfeld was able to convert the value into concentration of beta lipoprotein.

The precipitation of beta lipoproteins as polyanion complexes, employing sulfated mucopolysaccharides extracted from aortic tissue (1) and also with gelatin (2), has been reported by Amenta and Waters in 1960. Florsheim and Gonzales (21), in comparing ultracentrifugal separation and polyanion precipitation of beta lipoproteins, stated that the polyanion complex precipitation method proved to be adequate in the measurement of serum beta lipoproteins and that no denaturation of the protein resulted when a rice starch sulfate was employed. The use of such methods does provide a simple and inexpensive means of measuring the serum level of beta lipoproteins; but unfortu-

nately methods of precipitating other fractions of lipoproteins are still lacking or at least not reported at present.

It was mentioned earlier that one of the most notable methods of isolating serum lipoproteins was based on the principle of density. While performing an ultracentrifugal study of plasma proteins, McFarlane (44) observed a protein fraction which sedimented as a distinct peak upon the albumin fraction. Upon dilution this entity disappeared. The slower sedimenting peak was not always observable in his various studies, and since he observed it only occasionally, he identified the fraction as the "X Protein".

Pederson (50) further identified McFarlane's "X Protein" in 1945. After isolating quantities of the little-known fraction, he demonstrated that two distinct peaks could be observed when the ultracentrifugation was carried out in a salt medium. One peak was shown to have the same sedimentation constant as that of albumin. Upon increasing the solvent density in excess of 1.04, Pederson found that the "X Protein" would float to the surface of the tube during ultracentrifugation. On the basis of this observation, he postulated that the "X Protein" was a complex of albumin, globulin and plasma lipid.

It was the work of Gofman and other coworkers (26) of the Berkeley group which contributed greatly to the analysis of serum lipoproteins by the ultracentrifugal method. The

introduction of the S_f nomenclature by Gofman provided a means of characterizing lipoprotein fractions by their floatation rates in a solvent of density 1.063. The S_f rate is expressed in the same units as the usual sedimentation constant, except that the data are not reduced to values in water at 20 degrees centigrade and the negative sign is omitted. The S_f value depends upon the density, shape and size of the lipoprotein molecule.

Havel (29) later adopted the analytical ultracentrifuge study to differential ultracentrifugation, whereby lipoprotein fractions were isolated selectively by floatation in predetermined solvent densities. The lipoproteins were separated according to the following densities: less than 1.019; this fraction includes the large molecular weight species referred to as chylomicrons, which are predominantly lipid in character; the second fraction exists between density 1.019 and 1.063 and are the beta lipoproteins; the final fraction is composed of the alpha lipoproteins which are characterized by a density range of 1.063 to 1.21.

The high density fraction was further subdivided into two classes; a density 1.063 to 1.125 and also a density 1.125 to 1.21. The latter group was characterized by a greater amount of protein and little or no cholesterol. The protein fraction whose density is greater than 1.21 was shown to contain ten to fifteen per cent of the serum phosphorous, which was non-dialyzable, and to exhibit an electrophoretic migration with

alpha-1-globulin and albumin in starch electrophoresis.

Mention of electrophoresis has already been made in the study of serum lipoproteins. Indeed Macheboeuf employed this means in characterizing his horse serum lipoprotein fraction. Blix, Tiselius and Svensson (9) reported that considerable amounts of lipid were associated with the alpha and beta globulin fractions of normal human serum in 1941. They also found small amounts associated with albumin and gamma globulin fractions.

An electrophoretic study of ultracentrifugally separated lipoprotein fractions has been reported by Rezold, de Lalla and Gofman (51), in which fractions, isolated at densities 1.063 and 1.21, were determined. After staining the ionograms for lipid and protein, it was demonstrated that alpha and beta lipoprotein fractions were both contained in the more dense solvent; while beta lipoproteins and chylomicrons comprised the fractions in the less dense medium.

Other investigators have employed the use of electrophoresis in determining the amount of various lipid components present in the lipoprotein fractions. Nury et al (47) and Bloomberg and coworkers (11) measured the cholesterol content of lipoprotein fractions; while Chapin (15) investigated the phospholipid content in normal and atherosclerotic subjects.

CHEMICAL COMPOSITION OF SERUM LIPOPROTEINS

The lipoproteins found in the plasma of humans, as well

as in other animals, are not homogeneous entities; and are very similar to the gamma globulins in this respect. It is well, perhaps, to consider the lipoproteins as various families with a more or less similar composition among members of each family, but differing greatly from members of another family of lipoproteins.

In the preceding section it was shown that the fractions of lipoproteins varied greatly in their physical properties; it is not surprising therefore that other differences such as amino acid composition, lipid content, molecular weight and binding phenomenon will also be different.

It must be realized that the composition of the intact molecule with respect to the quantity and type of lipids, such as: triglycerides, phospholipids, cholesterol, both free and esterified, and non esterified fatty acids, will exhibit a profound effect on that molecule's solubility and density. Further consideration must be given to the amino acids involved, for these and free phosphate groups of phospholipids will effect the overall or net electronegativity of that molecule which is the most singularly important cause of their mobility during electrophoresis.

Compositional studies have been conducted by many investigators and a summation of their findings with regard to the properties and components of five plasma lipoprotein fractions are presented in Table I (48).

TABLE I

PROPERTIES AND COMPOSITION OF THE FIVE LIPOPROTEIN FAMILIES

Density at 25°	S _f Value at Density 1.063	Electro- phoretic Mobility	Conc. in plasma (mg. %)	Peptide	Phospholipid	Cholesterol Alcohol	Cholesterol Esters	Triglyceride
0.94	- -	*	*	2	7	2	6	83
0.98	400-10	B ₁ (or A ₂)	150	9	18	7	15	50
1.03	10-2	B ₁	320	21	22	8	38	10
1.09	-1	A ₁	80	33	29	7	23	8
1.14	-2	A ₁	380	57	21	3	14	5

* Variable values

It will be noted that with increasing density there is a concomitant increase in peptide and phospholipid content, while the content of triglyceride is decreased. The concentration of free cholesterol and its esterified form is associated with the beta and lighter alpha lipoproteins for the greatest part. A more extensive treatment will be given to cholesterol and triglyceride in the next section, which will discuss the subject of transport and metabolism.

The amino acid composition of the foregoing lipoproteins has been reported by Shore and Shore (57) and is presented in Table II. It was found that beta lipoproteins of density 0.98 and 1.03 have identical amino acid patterns, as is also true of the alpha lipoproteins of density 1.09 and 1.14.

The appreciable concentrations of leucine and phenylalanine in both lipoprotein fraction may be of significance since they offer an attraction for the aliphatic residues of the lipid moieties.

Shore (55) has further investigated and reported the terminal amino acid groups of the plasma lipoproteins. He reported that in alpha lipoproteins of both density 1.09 and 1.14 one mole of aspartic acid per 100,000 grams of peptide was found as the free amino terminal acid and threonine as the free carboxyl terminal acid. He further reported that the alpha lipoprotein of density 1.09 was shown to have two peptide chains of the above description, while the density 1.14

TABLE II
AMINO ACID COMPOSITION OF THE PLASMA LIPOPROTEINS

	<u>Moles Amino Acid per 10⁵ Gm Peptide</u>	
	<u>B-Lipoproteins</u>	<u>A-Lipoproteins</u>
	• 0.98 • 1.03	• 1.09 • 1.14
Glycine	33.4	36
Alanine	49.3	66
Valine	49.5	55
Leucine	131.7	135
Isoleucine	0*	0*
Phenylalanine	45.0	28
Proline	28.6	(28)
Tryptophan	**	**
Serine	51.1	55
Threonine	49.5	45
Half-Cystine	41.1	26
Methionine	0*	0*
Aspartic acid and asparagine	76.0	62
Glutamic acid and glutamine	73.5	131
Tyrosine		19
Lysine	94.0	57
Histidine	0*	17
Arginine	20	42
* Approximate value	** Value not cited in literature	

lipoprotein had only one such chain.

Molecular weights of 165,000 to 195,000 have been reported for the 1.14 lipoprotein, while a molecular weight of 365,000 to 435,000 has been reported for the 1.09 lipoprotein.

Shore has also reported one end group analysis of the beta lipoprotein fractions. In the density 1.03 protein two N-Glutamic acid and C-Serine peptide chains were described, in which each chain was reported to have a molecular weight of about 380,000. Molecular weight of the intact, anhydrous molecule was estimated to be between 1,300,000 and 3,200,000.

The lighter beta lipoprotein of density 0.98 was studied again by Shore and an end group analysis revealed the presence of at least an N-Serine and C-Alanine peptide chain with a maximum molecular weight of 12,000. The lipoprotein was purported to have a molecular weight ranging from 50,000,000 to 250,000,000, which certainly reflects a great deal of heterogeneity in this group.

Estimates of lipoprotein molecular weights have been determined by employing such methods as osmotic pressure, sedimentation studies from ultracentrifugal procedures and light-scattering.

It is apparent from the chemical composition of the beta lipoprotein that the lipid content represents approximately 75 per cent of the anhydrous weight of the molecule. Surgenor (58) has suggested an interesting postulate to the structural

aspects of the surface of these lipoproteins. On the basis of the peptide chain dimensions and assuming that such chains exist in monolayer form, he has calculated that an area of one-half the total lipid surface is covered by peptides. The remaining area of lipid surface is then postulated to be composed of polar groups from phospholipids. Alpha lipoproteins, on the other hand are predominantly protein in nature and are imagined to be composed of an entire outer surface of peptide material, thus completely covering the lipid material of this molecule. Such a suggestion seems plausible on the basis of the lipoprotein's solubility in aqueous solutions.

BIOCHEMICAL ASPECTS OF HUMAN SERUM LIPOPROTEINS

In the previous section attention was directed to the static properties of lipoproteins, namely the basic chemical and physical properties. It is now of some interest to focus upon the more dynamic aspects of these complexes by placing regard upon such matters as synthesis, transport and metabolic function. It is in these areas that the question and significance of lipoprotein existence is realized, and also these areas provide a vital category by which lipoproteins may be differentiated from other protein compounds.

Of first concern are the chylomicrons, the very low density lipoproteins (density 1.019, as used in this thesis), which apparently received this nomenclature from Gage and Fish (25) who

observed these particles in blood and lymph after the ingestion of a fatty meal. Because of their low density, chylomicrons are easily separated from raw serum or lymph by centrifugation at 10,000 g. They pack as a butter-fat layer at the top of the centrifuge tube. The apparent and exclusive site of chylomicron formation is in the intestinal mucosa, at which locus the digestion (hydrolysis) and absorption of lipids occur. The large molecular weight triglycerides (triglycerides of long chain fatty acids), cholesterol and its esters and carotenoids are incorporated with a relatively small amount of protein to form the chylomicron particle, whose size ranges between 0.5 and one micron. From this area the chylomicrons are transported by way of the lymphatic system to the thoracic duct. From this juncture the chylomicrons are passed into the vascular blood system.

Shortly after a meal consisting of a significant lipid content, one may observe a lactescence within the blood serum. Within a few hours, however, the milkiness disappears; this phenomenon is referred to as the "clearing factor". Some time after the discovery by Hahn (28) that injections of heparin caused a rapid disappearance of postprandial lipemia, other investigators (3) demonstrated that the heparin injection had promoted the appearance of the clearing phenomenon which could in effect "clear" the lipemia of plasma and artificial triglyceride emulsions, both in vivo and in vitro. Later Shore, Brown and Korn (56,13,34) investigated this mechanism and demon-

strated that it was in fact a lipoprotein lipase.

Korn further established very conclusively that an activating mechanism is necessary for the hydrolysis of triglyceride. This fact is very relevant since a basic review of thermodynamic principles regarding reaction states reveals the fact that any substance which will undergo a degrading reaction (i.e organic catabolism) must first be brought to an excited state from which the continuation of the reaction will proceed spontaneously. The excited state represents a dynamic level of internal energy of a particular substance and exceeds both the original energy within that substance and also the internal energy level of the product/s. Such an excited state is achieved within a substance by certain energy factors existent within the entire system, the most significant of which is the portion of energy released by the reacting substance during the course of reaction to the product/s.

It must be further borne in mind that the rate of reaction is a very important factor especially so within biological systems in order to provide for the metabolic necessities of the entire organism. A number of variables control the rate of a chemical reaction; among them being: concentration of reactants and products, temperature and acidity of the reacting medium. The inclusion of a catalytic reagent is likewise a factor in determining the rate of a reaction, and it is with this factor that Korn established his conclusion on triglyceride hydrolysis.

During the investigation of artificial triglyceride hydrolysis, employing coconut oil emulsions, it was noted that the addition of a small amount of normal serum resulted in a rate of triglyceride hydrolysis equal to that of lipoprotein triglyceride hydrolysis (chylomicron and low density beta lipoprotein). By fractionating the serum, Korn was able to isolate the catalytic agents responsible and determined that they were both alpha and beta lipoproteins.

His investigation, based on in vitro studies, then describes an interaction of triglyceride with a lipoprotein, either alpha or beta, to form a complex which in turn is acted upon enzymatically by a lipoprotein lipase to form the products of hydrolysis, namely: mixtures of glycerol, fatty acid and consequently the liberation of the lipase and the catalytic lipoprotein. The overall reaction proceeds through stepwise processes, involving sequential hydrolysis of the triglyceride into di- and monoglyceride forms. A necessary adjunct for a continuous reaction is the inclusion of fatty acid acceptors, which may be other enzyme systems in the tissues.

Although Korn has demonstrated the activation of artificial triglyceride emulsions by employing lipoprotein fractions of normal serum, he does not interpret this as being the formation of chylomicrons. And even though such synthetically prepared complexes immitate the same hydrolytic reaction that chylomicrons and other low density lipoproteins undergo, it is un-

reasonable to presuppose that chylomicrons are in fact aggregates of high density lipoprotein with triglyceride, cholesterol and phospholipids received from exogenous sources. This is so in light of the amino acid studies of the peptide chains comprising the various lipoprotein fractions. The amino acid content of the protein moieties of the low density beta lipoproteins is substantially different than that of the alpha counterpart, and hence it is not conceivable that the hydrolysis of triglyceride from low density beta lipoproteins will yield alpha lipoproteins. It is possible, however, that low density betas may be converted to a higher density by such hydrolysis, since the peptide chains of each type are similar in composition and also lipoproteins demonstrate similar immunochemical behavior.

Since only minimal amounts of lipoprotein lipase have been found in blood, it appears that the site of triglyceride hydrolysis is extravascular. The studies by Morris (46,23) and colleagues indicate that the liver is a principle organ in the removal of chylomicrons, since the appearance of plasma chylomicrons were observed in hepatic lymph, and also the removal of the liver resulted in a decreased rate of chylomicron removal. Bragdon (12), on the other hand, presents evidence that the distribution of chylomicron triglyceride fatty acid is extensive. This evidence suggests that chylomicron clearing occurs in areas which are rich in lipoprotein lipase, such as the liver, heart and adipose tissue. The site of such clearing with

triglyceride hydrolysis then could ostensible occur at the interface between the endothelium of capillaries and the cell wall.

The immediate fate of the fatty acids evolved from the hydrolysis of triglyceride is a matter of some importance at this point. Havel and Fredrickson (30) report these observations on the disappearance of chylomicron triglyceride fatty acid (palmitic acid- 1-C^{14}) from dog plasma. Comparisons were made between a palmitic acid- 1-C^{14} - albumin complex and chylomicrons which contained triglyceride with the labeled palmitic acid as one of the esterified fatty acids. Both preparations were studied by injecting solutions of each type into separate dogs and following the course of events through periodic blood samplings from the animals.

1) The disappearance of radioactivity from the low density fraction ($S_f=400$) which includes chylomicra and low density beta lipoproteins, appeared to follow a first order process and demonstrated a half-life of 10 to 25 minutes. Small amounts of radioactivity were found to be present in $S_f<17$ fractions, to which no particular significance could be given.

2) A rapid rise and then a fall in the specific activity of the plasma unesterified fatty acid occurred during the clearing process with no net increase in the actual unesterified fatty acid (UFA) concentration. The UFA-albumin complex also acted similarly in showing a rise and fall in specific activities. The authors first conclusion was that chylomicron

micron triglyceride was being hydrolysed very quickly and their constituent fatty acids largely retransported in the plasma. It appeared that only a very rapid turnover of plasma UFA would permit such loads to be transported. In a later series of experiments, however, the disappearance rates were followed over a longer period and it was indicated that three rate processes were present. The data from these experiments could be interpreted as fitting a situation in which the plasma UFA is in rapid equilibrium with a large body pool of fatty acids which appear certainly to be intracellular and also to be heterogeneous in composition. A major and irreversible pathway to CO_2 by oxidation is represented.

It is known that all exogenous cholesterol is carried to the blood stream by way of the thoracic duct lymph (8,16). During the absorption of cholesterol in the small intestine, a large portion of the compound is esterified by the action of an esterase. After absorption and esterification up to 97 per cent of the total chyle cholesterol has been found to be associated with the chylomicron fraction (49,21). In a labeled cholesterol experiment Fredrickson further reports that of the total cholesterol found in lymph 55 per cent is associated with the chylomicron fraction, while 88 per cent of newly absorbed labeled cholesterol has been determined to be associated with this same fraction; suggesting that newly absorbed cholesterol is not derived from a pool common to all

lymph lipoprotein fractions. Much of the radioactivity which was found to be associated with the higher density lipoproteins in the lymph can be explained on the basis of cholesterol exchange, which process will be explained a little later in this section. The data then suggests that chylomicra are the carriers of cholesterol in the early stages of absorption and transport and not the higher density lipoproteins.

The fate of exogenous cholesterol which enters the blood system in chylomicrons and very low density lipoproteins has been investigated by Fredrickson and Havel (29). Labeled chylomicrons containing C^{14} cholesterol were injected directly into the circulating blood system of dogs and serial determinations made on the plasma. The results showed a one-half decrease in the radioactivity of the chylomicron fraction within a period of 20 minutes. Also evident in the study was a sharp increase in the specific activity of free cholesterol associated with high density lipoprotein (density 1.063); this event occurred shortly after the injection of the labeled chylomicrons and continued for a period of 24 hours at which time the specific activity had declined until it was of no significance. Likewise there was an appearance of low level activity within the esterified cholesterol associated with high density lipoprotein fractions after a period of two hours from the time of injection. The above study clearly indicates that a marked difference exists in the rate of exchange of

chylomicron cholesterol to the free and esterified cholesterol of high density lipoproteins.

The observation was pursued further by investigating the exchange phenomenon between labeled chylomicrons and whole dog plasma in an in vitro study. The results confirmed the earlier observation, for indeed the exchange occurred with a rate up to nine times faster in the free cholesterol associated with higher density lipoproteins.

Gould (20,27) has demonstrated that the equilibration of labeled free cholesterol between plasma, liver and red cells is complete in a matter of a few hours. It has also been demonstrated by Gould and associates that exchange of free cholesterol occurs between high and low density lipoproteins in the S_r 0-17 fractions.

The rapid rate of cholesterol transfer from chylomicra to higher density lipoproteins suggest a divergent pathway from that of triglyceride which was discussed earlier. The work performed by Michaelis (24), Gould (20) and Chaikoff (31) indicates that the liver is primarily responsible for both the synthesis of much and the removal of practically all of this lipoprotein cholesterol, assuming that the cholesterol has been transferred to and is being transported by the beta lipoproteins, which fact appears apparent at this time on the basis of the isotopic data just presented.

The site of lipoprotein synthesis has been carefully

investigated by Radding and Steinberg (52). The serum lipoproteins (density 1.063 - 1.21) were compared to those lipoproteins which were synthesized by rat liver slices during an in vitro study. The results of a radiographic study of the hydrolysates from both sources indicate that they are identical to one another and thus establish the fact that the high density lipoproteins are synthesized by the liver. Some data was also collected which suggests that low density lipoproteins are also synthesized in the same fashion, but the data, per se, was not conclusive.

Work by Marsh and Whereat (38,39) has indicated that at least some of the low density lipoproteins secreted by the rat liver slices are identical with serum low density lipoproteins. Radding and Steinberg have also shown that a high serum level of lipoproteins is in fact due to overproduction. This fact became apparent in the course of study of lipoprotein synthesis by nephrotic liver slices. The rate of lipoprotein synthesis by nephrotic liver slices was faster than that by liver slices from normal rats.

CLINICAL ASPECTS OF SERUM LIPOPROTEINS

Lipoproteins of all classes have been closely investigated in a variety of disease states as well as in non-pathological states, and correlations made between lipoprotein levels and disease processes; even correlations as to prognosis have

been presented. The following review then will serve to describe some of the more notable investigations which have been undertaken within the past decade.

Perhaps the most noteworthy research involving lipoproteins was that of Gofman and coworkers (32) in 1951, who were concerned with the etiology of atherosclerosis. Their evidence indicates that there is no positive correlation between the high density class of lipoproteins ($S_f < 10$) and atherosclerosis. Large concentrations of this class in amounts 10 to 15 times normal showed no atherosclerotic tendency. In fact severe atherosclerosis was often observed with low concentrations of this fraction. A high degree of correlation exists between the S_f 10-30 class and atherosclerosis. In some groups of animals a good correlation between total blood cholesterol and the disease is also evident, however this is due to the fact that the S_f 10-30 molecules account for a large portion of the total cholesterol.

In human subjects Gofman reports that S_f 10-20 lipoproteins are positive in the correlation to atherosclerosis, although class S_f 12-20 represents a much better index. The studies among humans employed myocardial infarction as the sole indicator for atherosclerosis; since 90-95 per cent of these cases manifested a complication of atherosclerosis of the coronary arteries, which fact was attested to by autopsy reports. It is admitted by the authors that the

incidence of atherosclerosis does precede the onset of an infarction, and in fact it is estimated that between 30-50 per cent of the normal male population are actively developing arterial atheromas.

Gofman further reveals that an increase in concentration of the S_f 12-20 lipoproteins during the recovery of an infarction correlates well to a worsened prognosis, while a decrease in concentration diminishes the chances of a recurrence of an infarction. During the study of the infarction cases the only therapeutic measure undertaken was a restriction of fat intake.

Keyes (33) has commented on the above study by Gofman by stating: "It is entirely unjustified to attribute to S_f 10-20 molecules any specific virtue beyond that for simple cholesterol measurements for prediction of arteriosclerosis of the estimation of the activity of the arteriosclerotic process."

Bernfeld (6) applied his nephelometric technique in the study of a wide variety of disease processes. His investigation reveals that beta lipoprotein concentration increases with age and also that increases were found in cases with arteriosclerosis, both of cardiovascular and cerebrovascular nature. He also reports that hypertension, per se, is not associated with increased beta levels, but does indicate an increase when associated with the cerebrovascular disease.

Elevations of beta lipoproteins were demonstrated in

cases involving diseases of the kidney and liver, diabetes and hypercholesterolemia. No changes were observed with malignancy or anemia, arthritis, neurological disease or obesity. It was also determined that the correlation of cholesterol level to beta lipoprotein concentration was enhanced as the total cholesterol level rises.

Serum lipoproteins have been investigated in cases of biliary cirrhosis by Russ, Raymont and Barr (53), who report that only traces of alpha lipoproteins are present and the presence of normal betas can be inferred only by immunologic tests. Their presence, however, accounts for a relatively small portion of the enormously augmented plasma lipids. Most of the lipids are combined atypically with beta globulins to form low density aggregates which behave similarly to normal beta lipoproteins during ultracentrifugal floatation studies, but which are quite dissimilar in chemical analysis.

THEORY OF CHROMATOGRAPHY

The separation of solutes in a mixture is the main purpose served by chromatography and it is brought by several factors which are inherent in the chromatographic phenomenon, namely: surface adsorption, ion exchange and partition between solvents (10). It is the latter factor which is of primary concern in paper chromatography, the type used in this thesis. Consden (19) had determined that separation and resolution of

amino acids occurred more satisfactorily in systems utilizing partly miscible solvents. The overall principle of this technique involves saturating the paper or other similar supporting medium with water from the solvent:water atmosphere which exists within the chromatographic system. The cellulose fibers of the paper possess a strong affinity for water and not organic solvent. The water saturated paper, which is in vapor equilibrium with the atmosphere, may be considered as a stationary phase; while the organic solvent which flows through the paper may be considered as the moving or mobile phase.

The movement of a solute may be explained as follows: as the organic solvent moves into the area of a solute, a partition occurs between the two phases and consequently some solute enters the moving phase and proceeds further along the paper. When the moving solute arrives at an area of the stationary phase which contains no solute, another partition occurs with a resultant transfer to the stationary phase. This process continues and may be used to good advantage in separating mixtures of solutes. Those substances which have a greater affinity for the moving solvent phase may be expected to travel faster than those which have a lesser affinity, and as a consequence will demonstrate a greater separation.

The factor of ion exchange is also present and indeed some exchange must occur between solutes, which have ionic

character, and the polar constituents of cellulose fibers, as well as other impurities which may be impregnated in the paper.

The above concept is clear and simple; it provides a basic explanation of the mechanism involved in the chromatography of simple solutes. In describing events of a more complicated nature, such as lipoproteins, one must by necessity include additional factors within the stationary-mobile phases. It is necessary to account for the pH and ionic strength of the solvent system. The factor of pH will certainly determine the nature of the charge on amino acid residues within the protein moiety of the molecule and hence determine not only the protein's solubility, but will also influence the charge on the polar group of the cellulose fibers thereby affecting an attracting force. The ionic strength of the solvent solution will affect the overall molecular structure of proteins and consequently its solubility. Inclusion of the proper organic solvent will play the predominant role in fractionating lipoproteins by presenting a suitable solubilizing medium for the lipid moiety.

It must be mentioned that the separation of solute from a mixture is not the only requisite for a good chromatographic procedure; the resolution of the moving substances into compact spots or areas is of prime importance for analytical purposes. From The previous description of chromatographic

mechanism, it must be apparent that the principle lies in equilibrium, in which the solute is tending toward a steady state between the two phases mentioned. In achieving equilibrium it is necessary to control the environment of the entire system in order to provide constant conditions which will not upset the equilibrium process. The importance of an equilibrated atmosphere and paper with respect to the concentration of solvent and water is a paramount factor, and tantamount to this is a constant temperature throughout the entire system. The temperature factor is necessary in maintaining the proper humidity of the atmosphere. It will be recognized that these conditions can only be achieved in an isolated and equithermal area, and for these reasons sealed vessels are used with careful attention directed to the maintenance of a constant temperature. Both paper and atmosphere are allowed to equilibrate with one another in the presence of the solvent mixture for a sufficient period of time before a chromatographic procedure is conducted.

The choice of material to support the stationary phase is of great importance in the ultimate separation and resolution of the solutes. It has already been mentioned that cellulose fibers contain compounds which possess a polar nature, and these will influence moving ionic solutes. The problem of adsorption in the supporting medium is also of consequence, for indeed vander Waal forces are at play and will exert their

attraction for moving solutes. Electroosmosis also may contribute to these forces by establishing a potential gradient across the minute capillary walls of the paper as the aqueous solution of electrolytes pass through these capillaries.

The process of diffusion is in evidence, for the enlargement of solute patterns may be observed during the course of development. The phenomenon is limited, however, by forces of cohesion within the molecules of solute and also by the attracting forces within the supporting medium.

In order to promote separation and resolution of complex components (i.e. proteins), due consideration must be directed to the elimination of certain elements or compounds, present within the system, which may react chemically with the complex components to form products of such a nature that they drastically alter the original chromatographic properties of those components. An example of such a condition is the interaction of heavy metals (i.e. copper and mercury ions) with proteins to form a heavy metal salt. Solubility of such a compound is considerably less than that of the parent protein, and development of the former reveals a pattern with considerable back-trailing. The degree of mobility will be dependent upon the time and extent to which heavy metal interaction has taken place.

An important aspect of chromatographic analysis is the determination of R_f values. These values are merely numer-

ical expressions based upon measurements of physical behavior within the chromatographic system. R_f values represent additional indirect information concerning the nature of a solute molecule, and as such are similar to other behavioral measurements such as: melting point, refractive index, freezing point depression and light absorption. The R_f value is the ratio of "the distance moved by the band of solute" to "the movement of advancing solvent liquid" and is a constant for a particular set of conditions within the chromatographic system. The conditions which must be considered and maintained are: the kind and length of supporting medium, the type and concentration of solvent components, the original position of the solute with respect to the solvent front, the period of development, the method of chromatography (ascending or descending) and the temperature of the system. It is apparent that careful standardization is required for accurate and valid R_f measurements.

In order to appreciate the mechanics of the chromatographic phenomenon, certain mathematical expressions must be considered. Consden, Gordon and Martin (19) developed a mathematical expression of this phenomenon by measuring certain factors involved. Their hypothesis was based upon a chromatographic column which was considered as being divided into successive layers of thicknesses (plates) and that the solution issuing from each plate is in equilibrium with the mean con-

centration of solute in the non-mobile phase throughout the layer. The thickness of such a layer is termed "height equivalent to one theoretical plate" (H.E.T.P.). It is assumed that the diffusion of solute from one plate to another is negligible and also that the partition of solute between the two phases is independent of its concentration and of the presence of other solutes. The following terms define the factors to be considered.

h = Height equivalent to one theoretical plate

A = Area of cross section of the column

A_S = Area of cross section of the non-mobile phase

A_1 = Area of cross section of the mobile phase

A_I = Area of cross section of the inert solid; i.e.

$$A = A_S + A_1 + A_I$$

v = Volume of solvent used to develop the chromatogram

α = Partition coefficient equals grams of solute per milliliter of non-mobile phase per gram solute per milliliter of mobile phase at equilibrium.

$$V = h(A_1 + \alpha A_S)$$

R = Ratio of movement of position of maximum concentration of solute to the simultaneous movement of surface of developing fluid in the empty part of the tube above the chromatographic column.

r = Serial number of plate measured from the top of column

If r is the number of the plate containing the maximum concentration of solute, its distance from the top of column is rh , but:

$rh = hv/V$, i.e. position of maximum concentration has moved a distance hv/V directly proportional to the volume of solvent used to develop the chromatogram.

$$\text{If } R = \frac{vh/V}{v/A} = \frac{\text{distance of solute}}{\text{movement of surface of liquid}}$$

$$R = \frac{Ahv}{hv} \cdot \frac{1}{A_1 + \alpha A_S}$$

$$R = \frac{A}{A_1 + \alpha A_S}$$

However R is not conveniently measureable in paper chromatography, so a new symbol, R_f , is introduced by the authors.

$$R_f = \frac{\text{Movement of band}}{\text{Movement of advancing front of liquid}}$$

LeRosen (35), while working independently of the above authors, simultaneously introduced an expression for the above ratio. The symbol R as presented by LeRosen is identical to Consden's R_f .

Therefore:

$$R_f = \frac{RA_1}{A} = \frac{A_1}{A_1 + \alpha A_S}$$

The useful partition coefficient, alpha (α) may be isolated from the above:

$$\alpha = \frac{A_1}{R_f A_S} - \frac{A_1}{A_S} = \frac{A_1}{A_S} \left(\frac{1}{R_f} - 1 \right)$$

The term A_1/A_S is equal to the ratio of volumes of solvent and water phase in chromatograms. Assuming a given water content of the paper, A_1/A_S may be deduced from the ratio of

weight of dry paper to that of the developed chromatogram.

In discussing chromatographic principles and mechanisms, attention has been centered upon paper chromatography for the most part. In conventional paper chromatography two methods enjoy wide popularity, namely: the ascending and the descending techniques. Essentially the two are similar in overall principle; both operations function by reason of capillary action, but in the descending method solvent flow is aided by gravitation, while the ascending method functions against this same force.

A system which provides a wide margin of control is that of column chromatography. This system has gained precedence in the separation of solute mixtures due to the finer separation and resolution of solutes and also to the ease in recovering solute fractions. This method while providing the above advantages is inherently slower in operation, primarily due to the slower rate of solvent flow. The solvent flow factor is one that is controlled directly by solvent feed mechanisms which can be readily adjusted to any given rate position. Glass columns can be fabricated to any length and bore, and can be packed with a wide variety of supporting mediums, including those which possess ion exchange properties. Particle size of the medium may be varied to fit the immediate need of the procedure, and also solutions and buffers may be readily interchanged during the course of development.

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in order to enhance or retard mobilities of solutes.

A major innovation of the paper chromatographic method provides the means of investigation for the analytical treatment of human serum lipoproteins in this thesis. The innovation is characterized by a superimposed vector of force, specifically centrifugal force, which combines with the hitherto-mentioned force of surface tension, as translated into capillary action, to provide a vastly increased solvent flow through paper. The net result of this method is demonstrated in the increased speed of development of solute patterns.

The problems which were pursued in order to provide an adequate appraisal of centrifugally accelerated paper chromatography and also to ascertain the validity of an important dye-staining method were as follows:

1) To demonstrate that the R_F values of serum lipoproteins as fractionated by the centrifugally accelerated chromatographic method are compatible with those determined by standard or conventional descending paper chromatography.

2) To demonstrate that the color intensity of Sudan Black B dye:lipoprotein complexes, as prepared by a modification of an existent prestaining technique, is proportional to the concentration of lipoprotein.

3) To determine the per cent composition of alpha and beta lipoprotein by employing the technique of centrifugally accelerated chromatography and compare these values with those obtained by ionographic separation in buffers stabilized with paper.

CHAPTER II

MATERIALS AND METHODS

Pursuant to any scientific investigation, a concrete and reliable procedure must be established, standardized and maintained throughout the course of that investigation. This section describes those methods and materials which were employed in investigating the analysis of serum lipoproteins.

HUMAN SERUM

Human blood serum was procured from the Department of Biochemistry, Cook County Hospital through the courtesy of Mr. Alvin A. Dubin and Dr. Edward W. Bermes. Serum samples from at least 50 fasting patients were pooled for study. Samples from patients with known or suspected liver pathologies were excluded from the pool in order to achieve a uniform mixture which would approximate a normal concentration of serum components, particularly the lipoprotein fractions. Fresh serum was fractionated by ultracentrifugation and stored in a deep freeze at 0-3 degrees C. until ready for use. A portion of the sample mixture was stored under refrigeration for use

in whole serum studies. The period of such storage was limited to only a few days in order to avoid possible denaturation.

PRESTAINING TECHNIQUE

Since the technique of Sudan Black B prestaining by McDonald and Ribeiro (41) proved satisfactory in the study of R_B values by Banaszak (4), this technique was used exclusively during this investigation. The original technique was modified in order to achieve a more intense stain in the lipoprotein pattern, as developed by centrifugally accelerated chromatography. The patterns developed by this method are much larger and more diffuse than those obtained from conventional paper chromatographic methods or paper ionography, and as a consequence lipoprotein patterns became less intense in color, thereby making detection of the fractions more difficult. The introduction of the modified technique provided a substantial improvement in staining qualities to the extent that complete identification of the lipoprotein fractions could be obtained.

Solubilization of Sudan Black (Harleco, Lt 36) in propylene glycol was found to be enhanced by introducing ethyl acetate as a primary solvent. Initially the modification consists in mixing 0.1 gm. of the dye with 1.0 ml. of ethyl acetate (Reagent Grade). This mixture is stirred for several moments until a thin pasty consistency is obtained. Finally 9.0 ml. of propylene glycol is added slowly with con-

stant stirring. This procedure produced a 1 gm. per cent solution of Sudan Black B.

The prestaining of serum lipoproteins is accomplished by adding 0.2 ml. of the one per cent Sudan Black B dye solution to 1.0 ml. of fresh human serum or to 1.0 ml. of an ultracentrifugal lipoprotein fraction. This mixture is allowed to stand for 15 minutes with occasional gentle agitation or stirring. After this period the serum-dye mixture is centrifuged for five to ten minutes in a clinical centrifuge (International Clinical Centrifuge, Model CL). A small amount of unreacted dye may be observed as a precipitate which is discarded just prior to their use. The samples of prestained serum were never stored, but were always prepared before an analysis.

ULTRACENTRIFUGAL FRACTIONATION OF SERUM LIPOPROTEINS

Chylomicra, alpha and beta lipoproteins of human serum were fractionated by the method of differential ultracentrifugation as outlined by Havel (29). During the preparation of the standardized salt solutions, particular attention was devoted to the elimination of heavy metal ions from those solutions. The laboratory supply of distilled water was redistilled in an all glass apparatus in order to eliminate copper ions which were found on appreciable amounts in the stock water. The redistilled water was employed in all phases of the salt

solution preparation and ultracentrifugal procedures. All equipment to be used during the course of the entire procedure was scrupulously cleaned and the redistilled water used as a final rinse.

All standard density solutions were prepared from a stock salt solution of density 1.346 gm./liter. The stock solution was formulated according to Havel by dissolving 153.0 gm. sodium chloride (Mallinckrodt, Reagent Grade) and 354.0 gm. potassium bromide (Mallinckrodt, USPP in a sufficient volume of redistilled water to provide a final volume of one liter.

In order to remove the remaining concentration of heavy metal ions, a dithizone extraction method was performed, as employed by Bermes (7). To the stock salt solution was added a saturated solution of diphenylthiocarbazone in carbon tetrachloride. After vigorously agitating the mixture, the metal-dithizone complex was separated from the mixture by decanting the carbon tetrachloride phase. Remaining dithizone was removed from the salt solution by five extractions with 500 ml. volumes of carbon tetrachloride. Remaining traces of carbon tetrachloride were removed by boiling the salt solution for 30 minutes.

Preparation of standard density solutions were made by diluting the stock solution with volumes of redistilled water as determined by the following formula:

$$(A \cdot Y) + (B \cdot Z) = (A + B) \cdot X$$

A = Volume of redistilled water

Y = Density of redistilled water

B = Volume of salt solution to be used

Z = Density of salt solution

X = Final density of diluted salt solution

The final density of the standard salt solutions was carefully checked by means of a 10 ml. specific gravity bottle; all weight measurements were performed on a Gramatic Analytical Balance and conducted at a temperature of 20 degrees C.

The above formula also served in determining the volume ratios of serum to salt solution. After determining the desired volumes of serum and salt solution, necessary to provide a final composite density, those volumes were carefully pipetted into a Lusteroid ultracentrifuge tube (15 ml.) and any remaining tube space filled with a standard salt solution of the final density. The tube filling procedure was carried in a method as employed by Bermes (7).

The filled tubes were capped and placed into the tube compartments of a Spinco Number 40 Rotor head and subjected to an average centrifugal field of force of 105,400 g for a period of some eighteen hours. This procedure was carried out in a Spinco Model L Ultracentrifuge at a chamber temperature of 12 degrees C. After the required time had elapsed the tubes were removed from the rotor head in order to remove the lipo-

protein concentrates which had floated to the tube surface as the supernatant.

An elaborate recovery method was employed utilizing a precision made tube slicer which provided complete and accurate tube sectioning in order to isolate the lipoprotein supernatant. After the slicing blade had pierced the Lusteroid tube at the desired position, the supernatant was withdrawn by means of a syringe. The contents of the lower section of the tube (infranatant) were also collected for further instrument fractionating.

The above methodology was employed in the two isolation procedures which are now to be described for the recovering of alpha and beta lipoproteins.

I. A set of six tubes, having a final density of 1.063, was prepared by adding 2.0 ml. of salt solution (density 1.346) and 10.0 ml. of fresh human serum; the remaining space was filled with another salt solution (density 1.063). These tubes were then centrifuged for the eighteen hour period, as mentioned above, and the supernatants collected. The fractions, thus collected, were comprised of chylomicrons and beta lipoproteins. The infranatant was also collected and reserved for Procedure I'.

II. Another set of six tubes was prepared and run simultaneously with Procedure I. The final density of the medium in this set was 1.019. The tubes were prepared by adding 2.0 ml. of a standard salt solution (density 1.085) and 10.0 ml. of the

human serum to each of the six Lusteroid tubes. The final volume adjustment was made with a salt solution of density 1.019. After ultracentrifugation was completed, chylomicra were collected as the supernatant, and the infranatant saved for Procedure II'.

I'. The combined infranatant volume from Procedure I was measured; potassium bromide (recrystallized three times, courtesy of Dr. E.W. Bernes) and redistilled water were added in the proportion: 4.50 ml. of infranatant to 1.12 gm. potassium bromide and 1.63 ml. of redistilled water. The final density of this preparation was 1.210, and after centrifugation the alpha lipoproteins were collected as the supernatant layer.

II'. The pooled infranatants from Procedure II were adjusted to density 1.063 by adding 9.0 ml. of the infranatant to 3.0 ml. of a standard salt solution (density 1.196). A salt solution (density 1.063) was used to fill the remaining space before capping. This procedure yielded beta lipoproteins in the supernatant layer.

A brief resume of the above procedures with regard to the type of lipoprotein fraction collected and the corresponding density range may be seen in Table III.

TABLE III
ULTRACENTRIFUGAL FRACTIONS OF SERUM LIPOPROTEINS

<u>Lipoprotein Fraction</u>	<u>Density Range</u>	<u>Procedure</u>
Combined chylomicra & Beta Lipoproteins	1.063	I
Chylomicra	1.019	II
Beta Lipoproteins	1.019 - 1.063	II'
Alpha Lipoproteins	1.063 - 1.21	I'

DESCENDING PAPER CHROMATOGRAPHY

The conventional method of descending paper chromatography, employed during this investigation, was essentially the same as that utilized by Banaszak (4) in his study of serum lipoproteins. Large sheets of Whatman 3MM filter paper (forty-six by fifty-seven centimeters) were cut into strips of such a width that a multiple number of migrant tracks (each track being five centimeters wide) could be accommodated. The filter paper, manufactured by Whatman Ltd. of England, was specially designed for chromatographic purposes. The grade of the paper is course and allows a fast filtration rate. Since the paper is much thicker, it provides a greater wet strength than its thinner counterpart, Whatman Number 1 paper. The strips, sectioned from the large sheet, were cut along a plane which was parallel to the machine direction of the paper.

The migrant tracks were ruled by pencilling a fine mark along the length of the paper. Another line marked seven centimeters from the top and perpendicular to the track lines, served as the point of origin for the migrant.

The solvent system used in this chromatographic procedure was isopropyl alcohol:Michalec buffer (40:60, vol./vol.). Preparation of Michalec buffer followed the formulation as directed by the author (45). The buffer solution contained 10.3 g. of sodium veronal (Mallinckrodt, USP), 0.04 g. of oxalic acid: $2\text{H}_2\text{O}$ (General Chemical Co., Reagent Grade) and 0.61 g. of citric acid $\cdot \text{H}_2\text{O}$ (Baker Chemical Co., Reagent Grade) dissolved in distilled water. The final volume was adjusted to one liter by adding more distilled water. The measured pH of the solution coincided very well with the calculated pH of 8.6; the ionic strength of the solution was calculated to be 0.06. This buffer was used exclusively throughout the course of the paper chromatographic study.

In order to prevent denaturation of lipoprotein samples during chromatography, it was necessary to first completely saturate the paper and equilibrate it to the atmosphere in the chromatographic vessel for a period of at least one hour prior to the application of the lipoprotein sample.

A large, heavy glass cylinder, sixty centimeters high and thirty centimeters in diameter, and a heavy glass lid of the same diameter comprised the enclosure of the apparatus.

A stainless steel structure supported two glass half cylinders which contained the developing solvent, and with a glass rod in position secured the chromatographic paper in its hanging position. A circular glass dish was provided which served as an evaporating dish in which developing solvent could equilibrate with the enclosed atmosphere. The under edge of the glass lid was lubricated with stopcock grease in order to achieve an airtight integrity within the jar. A large shipping carton was used to insulate the jar from any air drafts or sudden temperature changes in the room.

After the saturated paper strip had been equilibrated, the prestained lipoprotein sample was carefully pipetted on the origin marking in the form of a streak in order to prevent the migrant from running. In most instances twenty microliters of sample was used, as this quantity provided a satisfactory stain intensity in the overall developed pattern.

A sufficient quantity of the isopropyl alcohol:Michael buffer was added to the half cylinders and the time noted; the cover was set in place and the chromatography continued for two hours. At the end of this time the developed chromatogram was carefully removed from the apparatus and dried in an oven at a temperature of 110 degrees C.

Since the prewetting of the paper strip precluded any possibility of locating the solvent front, it was necessary to resort to a marker method established by Banaszak (4) which

would serve as a secondary reference in place of the solvent front.

A 0.1 gm. percent solution of bromophenol blue (tetra-bromosulfonphthalein, National Aniline, Lot 13796) was prepared by dissolving 0.1 gm. of the dye in 100 ml. of distilled water. In practice the amount of the bromophenol dye solution used was equal in quantity to the volume of prestained lipoprotein sample employed. This methodology necessitated a revision in R_f terminology, since the distance transversed by the migrant band was equated to the movement of the dye band, instead of the solvent front. A new expression, R_B , was introduced to symbolize the above ratio (43). The distance of migration for both the lipoprotein and the dye solution was measured from the point of origin to the leading edge of the respective migrant band.

PAPER IONOGRAPHY OF SERUM LIPOPROTEINS

The use of paper ionography was employed in certain areas of this investigation as a primary source of reference, since the validity and accuracy of this method have already been established and mentioned in the first chapter.

All ionographic studies were performed in a Precision Scientific Ionograph, using Whatman 3MM paper strips as the conduction medium. These strips, measuring one-half inch in width, were fastened into the removable supporting frame of the

apparatus and then saturated with a veronal buffer solution (pH 8.6 and ionic strength 0.05). After blotting excess droplets of buffer from this unit, the paper was subjected to an equilibration period of one hour at a constant temperature of 25 or 30 degrees C. in the watercooled ionograph chamber and a constant voltage of 5 volts/centimeter applied. After equilibration the prestained lipoprotein samples were applied as a streak to the center of the paper. A ten microliter pipette was used for this purpose.

The duration of the electrophoretic separation was two and one-half hours, commencing from the time of sample application. The previous conditions of voltage and temperature were maintained during this period. After completion of the run, the paper ionograms were quickly removed and oven-dried at a temperature of 110 degrees C.

The preparation of the veronal buffer solution followed the formulation: 20.6 gm. of sodium barbital and 2.80 gm. of barbituric acid (Mallinckrodt, USP) dissolved in distilled water and adjusted to a final volume of one liter. The pH of this stock solution was measured and found to be 8.6; the ionic strength was calculated to be 0.10. Dilution of the stock solution with an equal volume of water provided the working solution of ionic strength 0.05.

DENSITOMETRIC DETERMINATIONS

Densitometric measurements of developed chromatograms and ionograms were taken in order to determine the relative compositions of alpha and beta lipoprotein fractions. This type of measurement provided a substantial degree of ease and accuracy as compared to existent elution techniques in which complete removal of Sudan Black B stain was more difficult.

The scanning method was performed with a component system which included:

- 1) A Bausch and Lomb monochromator having two adjustable slits, one at the light source inlet and the other at the outlet and also a variable wave length control in the visible field of the spectrum.
- 2) A tungsten bulb which provided white light and was powered by a standard six volt storage battery.
- 3) A Welch Densichron unit, containing a blue probe with maximum sensitivity at 400 millimicrons, which served to amplify the small potential generated by the photocell.
- 4) A Welch log converter unit, connected between the Densichron unit and the recorder, which converted the logarithmic signal into a linear potential.
- 5) A Minneapolis Honeywell Brown recorder which translated and graphed the amplitude of the incoming signal potential into a per cent of transmittance reading.

An electric motor drive assembly was incorporated in

the probe unit to feed the paper strip through the light field; the rate of drive was synchronized to the chart speed of the recorder.

All chromatograms and ionograms of prestained lipoproteins were measured at a wave length of 600 millimicrons, the absorption maximum of Sudan Black B dye, and at this setting light absorption was minimal in the presence of protein and salt, which both have maxima in the ultra-violet region of the spectrum.

In order to reduce the stray light of other wavelengths from that of 600 millimicrons, the smallest possible opening was selected at the output slit which was consistent with the sensitivity of the entire system.

Although Whatman 3MM paper was more desirable for chromatographic purposes, the greater thickness of this paper presented some difficulty in densitometric measurements. A greater amount of light had to be directed on the paper strip in order to make measurements possible. This could only be accomplished by sacrificing the smaller light opening for a somewhat larger one and by employing a greater sensitivity setting in the Densichron unit.

Zero optical density or 100 per cent transmittance was established by running the clear portion of the chromatograms, adjacent to the origin mark of the strip, through the light field as a part of the entire measurement. This procedure was

followed in each scanning.

After zero optical density had been established, the response of the system was checked by noting the proportionality of pen deflection between 0 and 100 per cent transmittance to several intermediate potentials which could be selected by adjustment with the Densichron range switch. When the system was in proper balance, the scanning of the paper strip was continued to completion and the graph reserved for planimetry measurement.

The areas bounded by the recorded tracing are proportionately related to the surface area and color intensity of the pattern of prestained lipoprotein chromatograms. By Beer's Law the foregoing is proportional to the concentration of dye present in the chromatogram. The area represented under the curve was determined by planimetry, employing a Kueffel and Esser compensating planimeter. The graph of each chromatogram was measured twice and the average value recorded. This value then is expressed in square centimeters per given volume of prestained serum. The ratio of alpha or beta lipoprotein area to the total lipid area yields the per cent composition of the respective fraction.

Since backtrailing and overlapping of protein fractions are normally encountered in paper chromatographic procedures, some compensation was provided by extrapolating a line from the peak of each fraction to the base line in an effort to approximate the area of that fraction if it alone were present. The

area of beta lipoprotein fractions includes all the surface from the origin to the extrapolated line; it includes the "chylomicron" fraction, which was considered to be of minimal concentration since the study was made on serum from fasting patients. Alpha lipoproteins comprise the remainder of the lipid area.

CHAPTER III

FRACTIONATION OF SERUM LIPOPROTEINS
BY CENTRIFUGALLY ACCELERATED CHROMATOGRAPHY

The application of centrifugal force to paper chromatography has been amply demonstrated and reported by McDonald and others (40,42). Separation of amino acid mixtures and accurate R_f measurements of these amino acids have been performed in relatively small periods of time as compared to existent conventional techniques.

A Labline Chromatofuge, Model 5080, was employed during the course of this investigation; it was manufactured by Labline, Incorporated, Chicago, Illinois. The overall dimensions of this instrument, constructed of stainless steel, are: 30 inches long, 25 inches wide and 36 inches high. It is powered by a one-third horsepower, constant speed electric motor; and the rotation controlled by a variable speed, V-belt drive assembly, which may be adjusted by means of a control knob located on the instrument panel. An RPM counter is located next to this speed control as a guide to rotational setting.

The chamber of the chromatofuge is square in construction and measures seven inches in depth; the length and width being the same as the overall dimensions. Access to this chamber is furnished by two hinged doors, constructed in such a manner as to provide a circular opening at the center when these doors are closed. The opening is six inches in diameter and is intended to accomodate the feed line from the pressure bottle assembly.

A heavy gauge stainless steel disk, approximately nineteen inches in diameter, is located at the floor of the chamber and is connected to a drive shaft from the power source. A stainless steel cover with a six inch circular opening at its center is connected to the base plate by five bolts. The Cover is cushioned on a rubber ring gasket which encircles the plate. The cover and plate comprise the head of the chromatofuge.

The circular opening of the head cover is sealed by a Bakelite disk which is connected to one of the overhead doors by means of spring connectors. This disk with a Teflon ring inserted on the contact face provides an efficient air-tight seal when the door is closed. A small opening in the Bakelite accomodates the insertion of the capillary feed tube.

The pressure feed system is comprised of a 500 ml. glass pressure bottle, a custom made glass delivery tube containing a Teflon valve and a ball-joint end. A capillary tube, having a complimentary ball-socket, was connected to the joint of

the delivery tube and secured in position by means of a compression spring clamp. A general practice of lubricating the surfaces of the glass joints with an inert silicone grease was followed. Vinyl tubing was securely wired to all connections in completing the delivery system. Pressure was maintained at two pounds throughout the entire system by means of bottled nitrogen gas.

Sheets of Whatman 3MM paper were cut into circles, eighteen inches in diameter. For simple separations and R_B values these whole circles proved to be sufficient for the purpose. Chromatograms, which were to be analysed by densitometry, required an additional paper modification, necessitated by an inherent phenomenon encountered during radial development. This phenomenon is characterized by an enlargement of the migrant pattern as the migrant and solvent proceed into increasing area of the circle's segment and is referred to as "fanning out". This occurrence prohibits the use of densitometry unless complicated geometric considerations are made. The problem is overcome by restricting the migrant path to a straight line. A paper strip, 1.27 cm. wide and 13 cm. long was designed and cut along each of the four quadrant radii with enough edging removed to isolate the strip from the main body of the paper. The overall appearance of this modification is that of a spoked wheel with each strip radiating from the hub at a distance 10 centimeters from the center. This simple innovation then

furnishes an effective means whereby longitudinal development may be obtained.

Prior to the chromatographic run an origin marking was circumscribed at a distance eleven centimeters from the center of either above-described circle. The paper form was then completely saturated with Michalec buffer, the same employed in conventional paper chromatography, and the excess blotted between two large sheets of filter paper. The wet paper was placed on the head plate and secured in position by means of a large nut provided for this purpose. The remainder of the apparatus was assembled and the motor turned on to a speed of 325 RPM. This speed was maintained for a period of five minutes in order to further remove the excess of buffer from the paper.

After these initial preparations had been completed, the machine was stopped and the cover removed from the head assembly in order to spot the prestained lipoprotein samples on the paper. In performing this operation, twenty microliter aliquots of sample were applied to the origin marking in a streak fashion by means of a micropipette. For those chromatograms designed for measurement of R_B values, aliquots of sample and bromophenol blue solution were streaked as a series in juxtaposition to one another at each quadrant of the paper circle. Those chromatograms, which were to be analysed by denitometry, required a single application of migrant on the origin marking of each strip within the modified circle. The cover was again

secured in position after the spotting was completed, and the capillary feed tube placed in position. The solvent feed and head rotation were started simultaneously. The solvent flow was regulated to a rate of 0.4 ml. per minute, and the rotation again maintained at 325 RPM. These conditions were continued for a period of thirty minutes, after which the developed chromatogram was removed and oven dried at 110 degrees C. The strips from the modified paper were cut from the main body and reserved for densitometric analysis.

CHAPTER IV

RESULTS AND CONCLUSIONS

The R_B values obtained by the method of centrifugally accelerated chromatography were compared to those values established by conventional paper chromatography and reported by Banaszak (4). These comparisons are presented in Table IV.

It must be pointed out that the R_B values of ultracentrifugal fractions of serum lipoproteins, as determined by the method of conventional paper chromatography, were based upon measurements of poststained chromatograms. There appears to be little significant difference between the results of either staining technique, when a consideration of the standard deviation is made.

The R_B values as determined by the two methods are in agreement with one another, with the exception of the chylomicron fraction. The difference in value in this case is somewhat appreciable and may possibly be explained by stating that the determinations were made on ultracentrifugal fractions of chylomicra which were quite different in composition. The chylomicra

micra and beta lipoproteins of very low density represent the most heterogeneous groups within the entire family of lipoproteins. Since these comparisons were made on different ultracentrifugal preparations, it is not surprising that this difference would result.

RELATIONSHIP OF STAIN INTENSITY TO LIPID:DYE CONCENTRATION

The results of stain intensity, expressed as area under the curve, to the relative concentration of the lipid-Sudan Black B complex are presented in Tables V and VI and graphically illustrated in Figures 1 and 2. The data in this table represents densitometric determinations of a single series of five prestained serum solutions ranging between 100 and 20 per cent of the original concentration. Dilutions were made with Michalec buffer (pH 8.6, ionic strength 0.06).

By employing Beer's Law the proportionality of the area under the curve to the relative concentration of the original prestained serum solution may be ascertained. A working equation may be derived from the following Beer's Law expression:

$$I_0 = e k c d$$

Wherein

$$I_0 = \text{Incident light}$$

$$I = \text{Light transmitted}$$

$$e = \text{Mathematical constant}$$

TABLE IV
R_B VALUES OF LIPOPROTEIN FRACTIONS

Source	Lipoprotein Fraction	Average R _B Value	SD*	No. of Trials
Method: Centrifugally Accelerated Paper Chromatography				
Prestained Human Blood Serum	Alpha	0.99	0.05	3
	Beta	0.50	0.10	3
Prestained Ultracentrifugal Fractions of Blood Serum	Alpha	1.04	0.03	3
	Beta	0.48	0.06	3
	Chylomicron	0.25	0.02	3

Method: Conventional Descending Paper Chromatography				
Prestained Human Blood Serum	Alpha	1.03	0.03	6
	Beta	0.55	0.03	6
Poststained Ultracentrifugal Fractions of Blood Serum	Alpha	0.97	0.04	3
	Beta	0.57	0.04	3
	Chylomicron	0.39	0.03	3

* Standard Deviation of R_B values

TABLE V

STAIN INTENSITY VS. LIPID:DYE CONCENTRATION

TEN MICROLITER ALIQUOT

Concentration of* Prestained Serum	Area Under Curve in cm ²	Average Area in cm ²	Log ₁₀ of Average Area
1.00	16.1 17.1 -	16.6	1.220
0.80	14.7 16.9 15.4	15.6	1.193
0.60	12.4 13.3 13.7	13.1	1.117
0.40	- 10.8 11.0	10.9	1.037
0.20	7.5 7.9 8.3	7.9	0.898

* Concentrations are expressed as decimals and are relative to the initial concentration of the prestained serum. Dilutions were made with Michaelis buffer (pH 8.6, ionic strength 0.06)

TABLE VI

STAIN INTENSITY VS. LIPID:DYE CONCENTRATION

TWENTY MICROLITER ALIQUOT

Concentration of* Prestained Serum	Area Under Curve in cm ²	Average Area in cm ²	Log ₁₀ of Average Area
1.00	33.8 33.1	33.5	1.525
0.80	32.2 29.9	31.1	1.493
0.60	25.4 25.7	25.5	1.407
0.40	- 21.8	21.8	1.338
0.20	13.7 14.0	13.9	1.143

* Concentrations are expressed as decimals and are relative to the initial concentration of the prestained serum. Dilutions were made with Michalec buffer (pH 8.6, ionic strength 0.06)

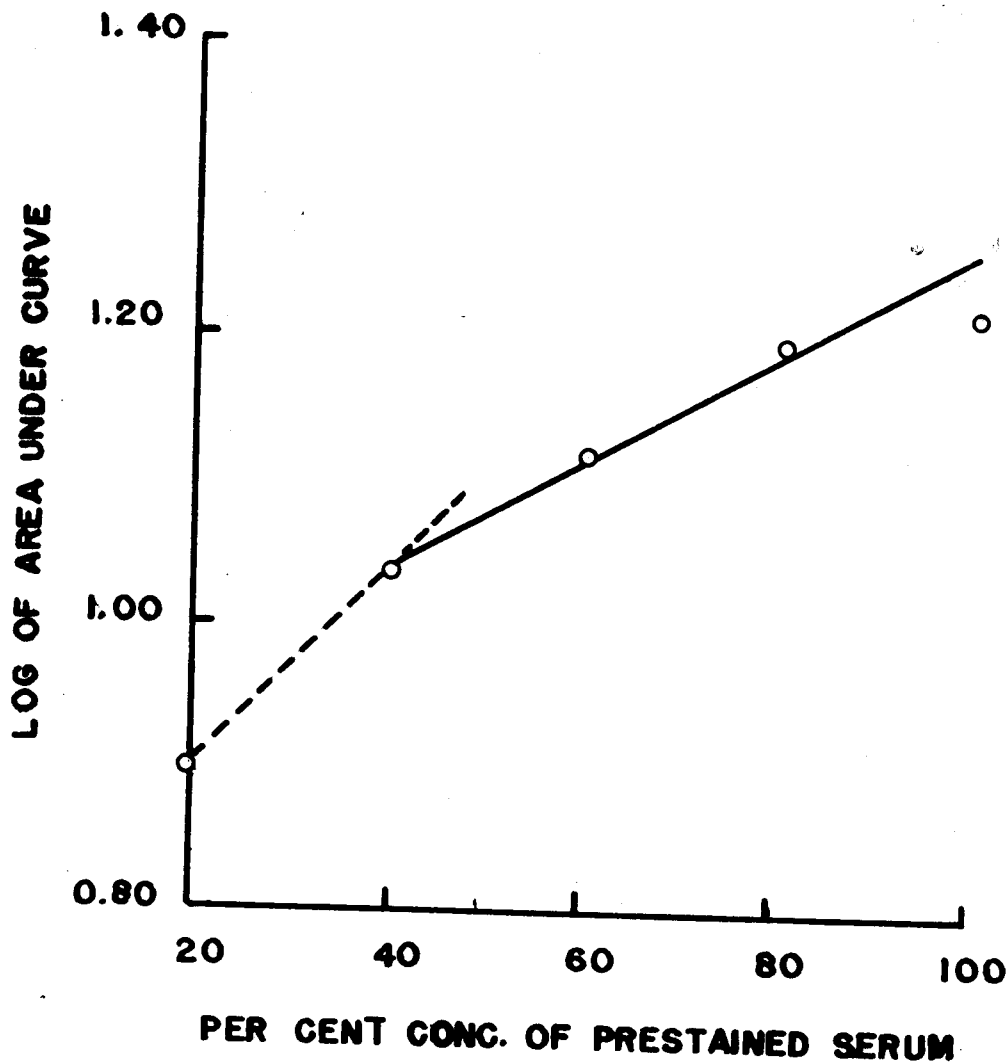


FIGURE 1

RELATIONSHIP OF LOG OF STAIN INTENSITY

TO PER CENT CONCENTRATION OF PRESTAINED SERUM

Ten microliter aliquots of five different per cent concentrations of prestained serum applied to Whatman 3 MM paper strips (one-half inch wide). Serum diluted with Michalec buffer.

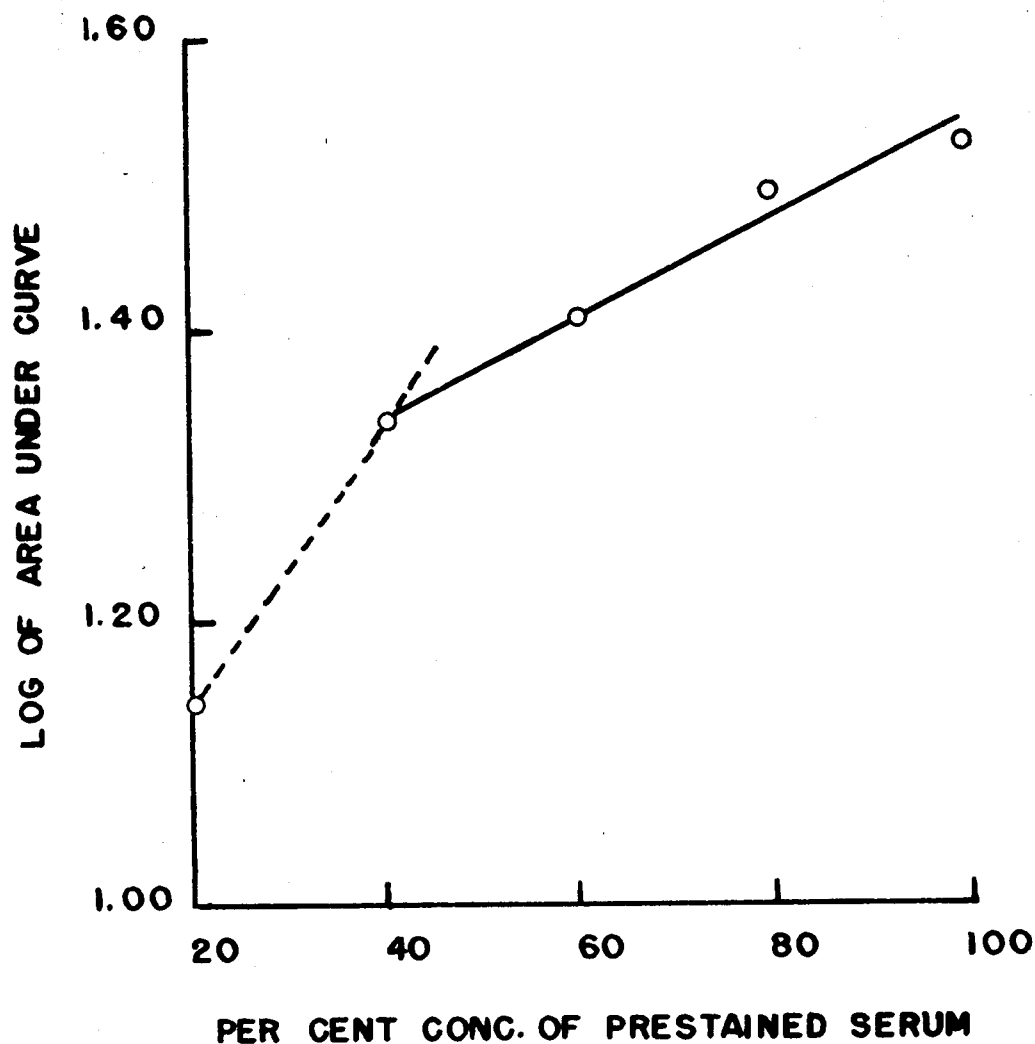


FIGURE 2

RELATIONSHIP OF LOG OF STAIN INTENSITY
TO PER CENT CONCENTRATION OF PRESTAINED SERUM

Twenty microliter aliquots of five different per cent concentrations of prestained serum applied to Whatman 3MM paper strips (one-half inch wide). Serum diluted with Michalec buffer.

k = Constant for the chromophore solution being studied

c = Concentration of the chromophore solution

d = Depth of the light field through the chromophore solution

$$\ln \frac{I}{I_0} = kcd$$

$\ln \frac{I}{I_0} = A$, which is the Absorption and is expressed in optical density units.

In densitometry optical density is directly related to the total area under the curve. By substituting the area under the curve in the preceding expression, the following equation is given:

$$\ln \text{Area (cm}^2\text{)} = kcd$$

For the determination of the constant (k) it is necessary to perform the calculations on a given volume of sample, which in this case will be ten microliters.

The actual concentration of the original lipid-dye complex is not known, but remains as a constant throughout these calculations. The depth of field (d) is also unknown, and it too shall remain as a constant. The value of (k) in each determination is expressed thusly:

$$k = \frac{\ln \text{Area}/10 \text{ mcl.}}{(\text{Rel. Conc.}) (c) (d)}$$

The values of (k) are presented in Table VII as well as the average areas per 10 microliters and the natural logarithms for these areas.

TABLE VII

BEER'S LAW CONSTANTS FOR
VARYING LIPID:DYE CONCENTRATIONS

Aliquot	Rel. Conc.	Area in cm ² per 10 mcl.	Ln Area	k*
10 mcl.	1.00	16.6	2.81	2.82
	0.80	15.6	2.75	3.44
	0.60	13.1	2.57	4.29
	0.40	10.9	2.39	5.97
	0.20	7.9	2.07	10.35
20 mcl.	1.00	16.7	2.82	2.82
	0.80	15.7	2.76	3.45
	0.60	12.8	2.55	4.23
	0.40	10.9	2.39	5.97
	0.20	7.0	1.95	9.75

$$* k = \frac{\text{Ln Area}/10 \text{ mcl.}}{(\text{Rel. Conc.}) (c) (d)}$$

It will be noticed that the values of (k) increase as the sample becomes more dilute. This indicates that the relationship of the area under the curve to the relative concentration is not directly proportional throughout the entire range. Although this is the case, the change in slope is not very extreme in the four samples of higher concentration and may be evidenced in the smooth curves of figures 1 and 2. The drastic change in slope for the 20 per cent entity is denoted by a dotted line.

The failure to obtain a proportional densitometric response for the solution of 20 per cent relative concentration was believed due to a deterioration of the lipid-dye complex within that solution. After the 20 per cent solution had been prepared, a suspension of black particles was observed to occur within that solution.

The observed phenomenon could not be explained on the basis of lipoprotein flocculation from a solution of insufficient ionic strength, since dilutions had been made with the working Michalec buffer, ionic strength 0.6g. If this were the case the use of such a buffer would have been ineffective in chromatography and as a consequence lipoprotein migration would not occur to any appreciable extent.

Furthermore, the formation of this suspension cannot be explained by citing ionic interaction as the cause, since the quality of the diluent was such as to exclude the possibility

of contaminants which could so react; and also such interactions would have occurred in all the diluted samples and not merely the 20 per cent entity.

A possible explanation for the observed suspension is that the lipoprotein-dye complex is in some way effected by extraordinarily large additions of aqueous solutions and as a consequence the complex is broken with the liberation of dye molecules which then aggregate to form these particles.

It will be noted that the corresponding areas of both aliquots are nearly equated to one another by a factor of two. This evidence demonstrates that the response of the densitometer is accurate and that measurements of varying amounts of stain may be made validly and with a reasonable degree of accuracy.

LIPOPROTEIN COMPOSITION

The values of six ionographic determinations and four determinations performed by the method of centrifugally accelerated chromatography are tabulated in Table VIII. A single source of serum was employed for all determinations. The values presented are expressed as the ratios of alpha or beta lipoprotein areas to the total lipid area. These values do not represent the actual ratios of these fractions, since the mode of measurement does not concern itself to the lipoprotein molecule per se, but rather concerns itself to the Sudan Black B - lipid complex. These values then are contingent upon the lipid com-

position of the respective lipoprotein molecule. No attempt has been made to introduce corrective numerical factors in order to derive values more indicative of actual concentration, since such procedures would be of the greatest difficulty, owing to the extensive heterogeneity of lipoprotein molecules, particularly those of low density.

Comparison of the values obtained by employing both ionographic and centrifugally accelerated chromatographic methods indicates quite clearly that both methods furnish reproducible results. Further comparison demonstrates that the results of centrifugally accelerated chromatography are quite compatible with those obtained by ionography. Since the validity of such ionographic measurements has been well established in lipoprotein study, the close agreement in value furnished by the method of centrifugally accelerated chromatography offers sufficient evidence for its validation as an investigative instrument for lipoprotein study.

The results of this investigation, employing centrifugally accelerated chromatography, appear not only significant but impressive. Although the accuracy and precision of this method do not compare as well as more elaborate analytical procedures involving strictly chemical techniques, they do compare favorably with those of conventional electrophoretic or chromatographic methods. It would be presumptuous to state that an ultimate in chromatography has been achieved; on the contrary,

TABLE VIII

DENSITOMETRIC ANALYSIS OF SERUM LIPOPROTEINS

Method	<u>Alpha Total</u>	<u>Beta Total</u>	<u>Standard Deviation</u>
Ionography of Prestained Serum	0.14	0.86	
	0.16	0.84	
	0.16	0.84	
	0.14	0.86	
	0.18	0.82	
	<u>0.17</u>	<u>0.83</u>	
	0.16	0.84	<u>0.02</u>

Centrifugally Accelerated Chromatography of Prestained Serum	0.18	0.82	
	0.21	0.79	
	0.18	0.82	
	<u>0.19</u>	<u>0.81</u>	
	0.19	0.81	<u>0.01</u>

several limitations in the methodology exist which indicate that further development should be applied. Some of the limitations and recommendations for improvement are as follows:

1. The paper supporting medium, although of the highest quality obtainable, proved to be a poor surface for obtaining compact resolution of lipoprotein fractions. Extensive backtrailing, encountered not only in centrifugally accelerated chromatography, but also in conventional chromatography and ionography, may be due in part to excessive migrant absorption in and adsorption to cellulose fibers of the paper. Research and development of synthetic materials and existing natural elements, which have been successfully utilized in column chromatography, could well be undertaken in order to furnish a product without the undesirable qualities of paper.

2. The instrument, as it was employed in this investigation, did not provide means of maintaining the equilibrium of the solvent atmosphere during the time of sample application. The removal of the cover from the head assembly permitted complete escape of the atmosphere, but since the volume of this atmosphere was relatively small, restoration of equilibrium did not require a great period of time. The design could be modified and small openings provided in the cover through which samples might be applied.

3. The solvent feed assembly proved to be troublesome on many occasions. Initially solvent flow rates of one to two ml.

perminute proved to be excessive for the development of lipo-protein fractions. The patterns resulting from such rates clearly indicated complete smearing had occurred and no acceptable separation had been achieved. D delivery pipette was fashioned from one of the capillary tubes by constricting the tip opening to the smallest possible bore which would permit the ejection of the employed solvent mixture in a small steady stream. The flow rate now could be reduced to 0.3 ml. per minute. This modification, however, was subject to occasional clogging which stopped the flow of solvent and consequently migrant development. Introduction of solvent to paper could be accomplished by delivery from an assembly employing a wick or capillary action; such a method would preclude solvent flow stoppage and would furnish the precise amount of solvent necessary for development.

In conclusion the use of centrifugally accelerated chromatography provides a means of obtaining good qualitative and semi-quantitative analysis of serum lipoproteins, which has been demonstrated to compare favorably with the conventional descending method and also ionography. The new innovation provides a considerable savings in time of development as compared to conventional techniques, and on this basis its use in research and routine chromatographic procedures is recommended, especially when multiple determinations are to be made or in situations requiring the most expeditious handling.

CHAPTER V

SUMMARY

A major innovation to paper chromatography, characterised by the addition of centrifugal force, has been investigated as an analytical method in the field of lipoprotein study, and as such was compared to the existing methods of conventional descending paper chromatography and ionography. The results of the comparisons proved to be satisfactory and indicated that the method of centrifugally accelerated chromatography provided a means whereby valid and accurate measurements of lipoprotein R_F values and compositions could be taken.

A modification to a present prestaining technique was presented and the validity of Sudan Black B dye-lipid complexes, as a measure of lipoprotein concentration, established by a densitometric method.

APPENDIX

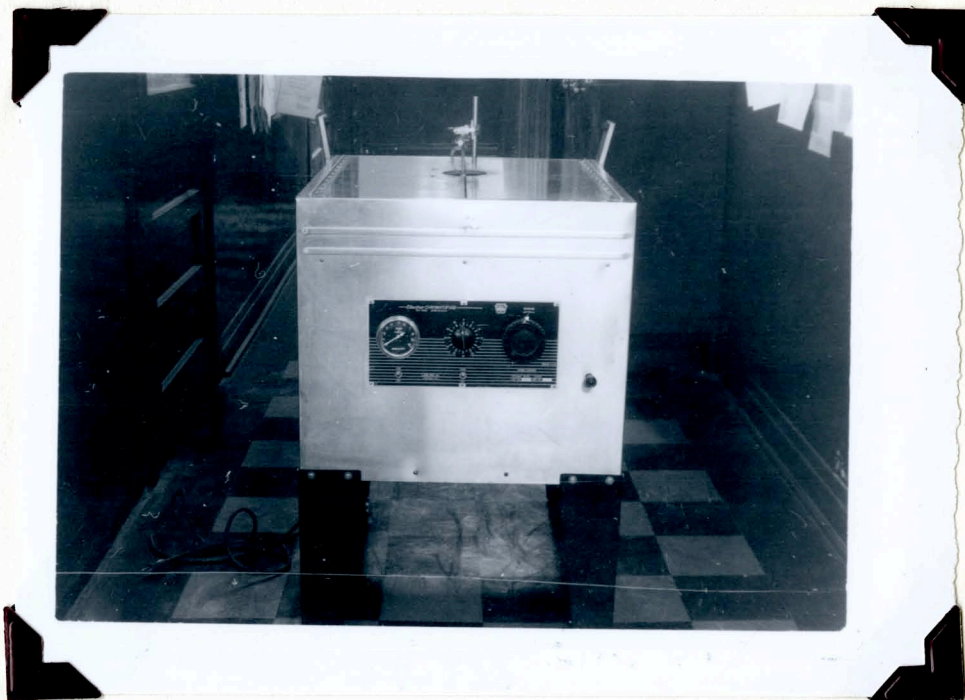


FIGURE 3

Photograph showing exterior aspects of Labline Chromatofuge with solvent delivery arm in place at the top center of unit. Note the RPM counter on the left of and the rotation control knob on right of instrument panel.



FIGURE 4

Photograph of Chromatofuge chamber showing the head plate with a modified 18 inch circle of Whatman 3MM paper in place. Note the "spoked wheel" design.



FIGURE 5

Photograph of a developed chromatogram showing the separation of serum lipoproteins on right of center and band of bromophenol blue on left of center. The alpha lipoprotein fraction is in the foreground, while the beta fraction is immediately behind.

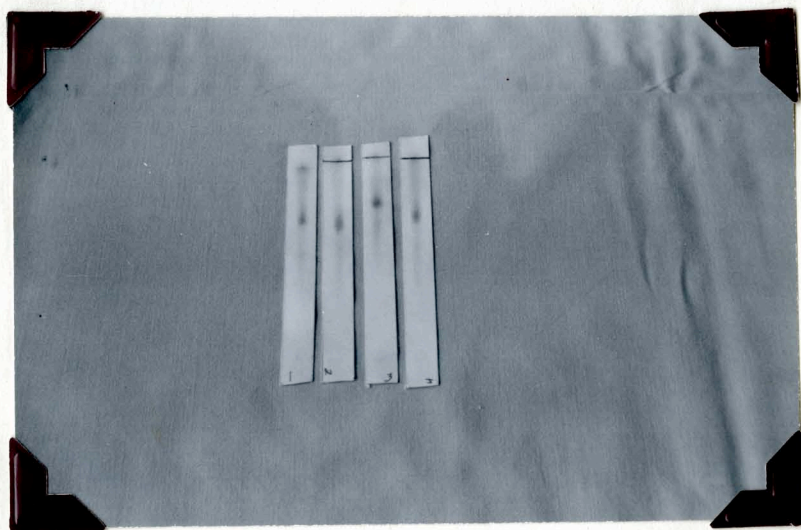


FIGURE 6

Photograph of four developed chromatograms removed from the modified circle. Note alpha lipoproteins in foreground and dense bands of betas immediately behind.

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APPROVAL SHEET

The thesis submitted by James Q. Kissane has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

January 10, 1963
Date

Hugh J. McDonald
Signature of Advisor